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13. ABSTRACT (Maximum 200 Words) <p>Improvement of hormone-based therapy in breast cancer and circumvention of its shortcomings is limited by the lack of detailed understanding of how steroids like estrogen work at a cellular and molecular level. The research supported by this award addressed the mechanism of action of estrogen action at its most fundamental level. Using newly-developed approaches, I investigated mechanisms of estrogen/estrogen receptor action on chromatin templates <i>in vitro</i> in order to better understand the role of chromatin in steroid-regulated gene expression. Specifically, experiments were proposed to assess the role of histone acetylation and high mobility group (HMG) chromatin proteins in estrogen receptor-directed transcription. Additionally, I sought to test whether <i>in vitro</i> transcription on chromatin templates could be used to address estrogen receptor action at nonclassical target genes. The chief accomplishment of these studies addressed the role of chromatin modification in activator-dependent transcription of chromatin templates. We found that the chromatin assembly extracts provided a critical factor and that acetyl CoA could restore activator-dependent transcription, implicating a critical role for chromatin modification in transcriptional regulation. However, other data suggested that acetyl CoA may account for only part of the stimulatory activity. Further experiments to address these findings are in progress.</p>				
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INTRODUCTION: This award supported sabbatical studies directed to elucidating molecular mechanisms of estrogen receptor action. Newly developed technologies employing the assembly of chromatin to serve as template for estrogen receptor regulated transcription in vitro were used to address the aims outlined in the original application. These goals were to address the influence of high mobility group proteins on the regulation of transcription by estrogen receptor, the role of histone acetylation in estrogen receptor action, and the mechanisms of estrogen receptor action at promoters with nonclassical response elements. The purpose of these studies was to acquire sufficient expertise in the chromatin assembly/in vitro transcription technologies to be able to initiate studies of this sort in my own laboratory. Although the specific aims were quite ambitious, progress was made on all three aims. The summary that follows details this progress. Most importantly, insights were gained that has opened new research questions and sufficient expertise was acquired to permit the continuation of further studies in my own laboratory upon my return to my home institution.

ANNUAL SUMMARY: My sabbatical leave supported, in part, through this award was spent at the University of California, San Diego, Department of Biology in the laboratory of Jim Kadonaga. Dr. Kadonaga has a stellar reputation for his pioneering work on the role of chromatin in the regulation of gene expression. Recently his laboratory developed the first in vitro transcription system in which gene induction by a steroid receptor recapitulated in vivo biology, opening the door to mechanistic studies on steroid receptor action that are not possible in living cells. It was to become familiar with this system that I arranged to conduct studies in the Kadonaga laboratory.

Preparation of S190 chromatin assembly extracts. The chromatin assembly/in vitro transcription assay has many components, each of which must be carefully prepared and tested. One of the first endeavors I undertook was to prepare S190 chromatin assembly extracts. These extracts are at the heart of the transcription assay for it is the assembly of the DNA into chromatin template that permits the proper regulatory properties to be reconstructed in vitro. S190 extracts are prepared from 0-6 hour *Drosophila* (fruit fly) embryos beginning with about 120 grams of material. This is accomplished by harvesting embryos from a dozen or more population cages of flies every 6 hours for 48 hours. I prepared two batches of S190 extracts. To assemble a template for in vitro transcription the S190 extracts are first incubated with purified core histones for 30 minutes at room temperature. This is for the

histone chaperone activity of the S 190 extract. The actual assembly is accomplished during a subsequent 4-hour incubation at 27° C. The quality of the S 190 extracts was assessed by analyzing the extent of the nucleosome array assembled on the plasmid template by micrococcal nuclease digests and plasmid supercoiling assays. Additional studies will be performed using these extracts in my laboratory at the University of Colorado Health Sciences Center.

Preparation of core histones. Assembly of plasmid templates into nucleosomal arrays requires the four core histones (H2A, H2B, H3, and H4). Core histones must be purified free from other nuclear proteins including linker histone Hi. I purified core histones from *Drosophila* embryos using a procedure developed in the Kadonaga laboratory. This gave me my first experience using an FPLC for protein purification. The purity of the preparation was confirmed by SDS-polyacrylamide gel electrophoresis. As with the SI 90 chromatin assembly extracts, these preparations of histones will be used in the additional studies that will be continued in my laboratory.

Preparation of HeLa cell transcription extracts. The transcription assay uses nuclear extracts from HeLa cells to provide many of the components of the transcriptional machinery. HeLa extracts were prepared beginning with a pellet of cells derived from 12 liters of HeLa cells. The procedure involves differential salt extraction and differential centrifugation. The preparation yielded about 5 milliliters of extract. The initial extract I made proved to be of poor quality and the preparation had to be repeated. The second preparation had good transcriptional activity. In addition to giving me experience with the preparation of HeLa extracts, these studies have provided the material to continue these studies upon returning to Colorado.

Preparation of tagged estrogen receptor and p300. Both of these proteins were overexpressed by infection of Sf~ insect cells with recombinant baculovirus vectors constructed in the Kadonaga laboratory. I purified estrogen receptor from extracts using FLAG affinity resins to selectively retain the FLAG tagged receptor. The coactivator p300 was expressed as a hexa-His tagged version and purified using commercial nickel resin. The addition of p300 to the transcription reaction greatly enhances the level of estrogen receptor-mediated induction of transcription. As above these preparations will continue to be of immediate use in continuing these studies.

Preparation of plasmids. Immediately before leaving Colorado I constructed two plasmids that I used in studies during the sabbatical leave. These two plasmids plus several others were purified from bacterial cell lysates by double banding over CsCl gradients in the laboratory at UCSD. These double-banded plasmids were used as templates for the studies that were performed and will continue to be of use in future studies.

In vitro transcription by estrogen receptor on classical and nonclassical templates. Once the necessary reagents were prepared I repeated previous work from the Kadonaga laboratory using the purified estrogen receptor to show ligand-dependence of estrogen receptor action on a classical, estrogen response element-containing promoter. Having done this, I tested whether estrogen receptor-mediated induction could be observed on a non-classical promoter where estrogen receptor interacts through a second protein instead of binding directly to sequence elements of the promoter. I chose the collagenase promoter for these studies. Estrogen receptor is thought to work by binding to the factor AP-1 which binds a site immediately upstream of the core collagenase promoter. Despite some hints that estrogen receptor-mediated induction could be seen, the effect was not large enough or consistent enough to believe. To see steroid receptor effects on the collagenase promoter probably is likely to require addition of purified AP-1 to the reaction as well. My laboratory will pursue this question further.

The effect of HMG proteins on transcriptional activation of chromatin templates by estrogen receptor. Additional studies addressed the role of high mobility group proteins 1 and 2 on estrogen receptor action. No effect of the addition of high mobility group proteins was observed on transcription. However, because these studies used crude S 190 chromatin assembly extracts and HeLa extracts, it is likely that high mobility group proteins were already present. Additional efforts in this direction will require the use of purified chromatin assembly extracts and purified transcription factors. It is now feasible to do such studies. Initial steps in this direction used purified chromatin assembly extracts and led to unexpected and interesting findings as detailed below.

In vitro transcription by progesterone receptor. Using purified progesterone receptor obtained from Dean Edwards at UCHSC and templates I made specifically for these studies, I demonstrated for the first time progesterone receptor-dependent transcription on a chromatin template. These studies also used for the first

time with steroid receptors a natural promoter derived from the mouse mammary tumor virus.

Chromatin assembled by a recombinant assembly system fails to support activator dependent transcription in vitro: implications for a role for histone modification. Probably the chief accomplishment of my experimental endeavors was the unexpected demonstration that when a new, fully recombinant system was used to assemble the chromatin templates rather than an S190 extract neither estrogen receptor nor a synthetic transcription factor Gal4-VP16 could subsequently activate transcription. A series of studies showed that the effect of S190 was on a step subsequent to assembly. I speculate based on this and data discussed below that a remodeling of the chromatin template does not occur in the absence of S 190. I showed further that much of the transcriptional activity could be restored by the addition of AcCoA. This is a particularly interesting finding since the role of acetylation of histones and other proteins is presently a topic of intense investigation. AcCoA is a substrate for the acetyltransferase enzymes that carry out the acetylation. However, close examination of several facets of my data suggest that S190 has an activity in addition to or instead of simply providing the AcCoA required for the activation of transcription. This subject will be pursued in my own laboratory. In addition, I plan to take advantage of my newly acquired expertise, reagents, and results to write grant applications to support pursuing these and other issues that can be addressed by exploiting the chromatin assembly/in vitro transcription system.

KEY RESEARCH ACCOMPLISHMENTS:

- Acquired the expertise to perform the techniques required for studies utilizing in vitro transcription by estrogen and other steroid receptors on chromatin templates.
- Prepared purified protein and functional extracts used for the described in vitro transcription studies as well as for use in future experiments that will build on the results obtained to this point.
- Evidence for in vitro transcription on natural promoter by the progesterone receptor.
- Evidence for a requisite role of Acetyl Coenzyme A in receptor-dependent and transcription factor-dependent transcription from chromatin templates.
- Additional data suggesting that factors in addition to Acetyl CoA may play a role in transcriptional regulation on chromatin templates.

REPORTABLE OUTCOMES:

1. Abstract poster presented at Keystone Symposium: Nuclear Receptors 2000 "Is chromatin modification or chromatin remodeling required for activator-dependent activation of transcription in vitro?" SK Nordeen, ME Levenstein, W Jiang, WL Kraus, JT Kadonaga
2. Abstract talk presented at Keystone Symposium: Nuclear Receptors 2000 "Chromatin structure and the Regulation of Transcription by RNA Polymerase II" X Huang, W Jiang, SK Nordeen, DV Fyodorov, ME Levenstein, V Alexiadis, JT Kadonaga
3. Presentation: The role of chromatin modification and chromatin remodeling in activator-dependent transcription. Talk given to Division of Medical Oncology, Univ. Colorado Health Sciences Center
4. Presentation: Chromatin remodeling and modification in the regulation of transcription by steroid receptors. Talk given to the Hormones and Cancer group of the University of Colorado Cancer Center
5. Presentation: The role of chromatin modification and chromatin remodeling in activator-dependent transcription. Annual Meeting of the Front Range Transcription Group.
6. Funding Applied for: NIH "Molecular Mechanisms of Steroid Hormone Action"

Transcriptional Analysis of Chromatin Assembled with Purified ACF and dNAP1 Reveals That Acetyl-CoA Is Required for Preinitiation Complex Assembly*

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To investigate the role of chromatin structure in the regulation of transcription by RNA polymerase II, we developed a chromatin transcription system in which periodic nucleosome arrays are assembled with purified recombinant ATP-utilizing chromatin assembly and remodeling factor (ACF), purified recombinant nucleosome assembly protein 1 (dNAP1), purified native core histones, plasmid DNA, and ATP. With this chromatin, we observed robust activation of transcription with three different transcription factor sets (nuclear factor κ B p65 + Sp1, estrogen receptor, and Gal4-VP16) added either before or after chromatin assembly. In fact, the efficiency of activated transcription from the ACF + dNAP1-assembled chromatin was observed to be comparable with that from naked DNA templates or chromatin assembled with a crude *Drosophila* extract (S190). With ACF + dNAP1-assembled chromatin, we found that transcriptional activation is dependent upon acetyl-CoA. This effect was not seen with naked DNA templates or with crude S190-assembled chromatin. We further determined that acetyl-CoA is required at the time of preinitiation complex assembly but not during assembly of the chromatin template. These findings suggest that there is at least one key acetylation event that is needed to assemble a functional transcription preinitiation complex with a chromatin template.

The regulation of gene expression at the level of transcription is a key control point for many cellular processes. In eukaryotes, the transcription of protein-coding genes by RNA polymerase II occurs in the milieu of chromatin and involves

the covalent and noncovalent modification of nucleosomes (for reviews, see Refs. 1–13). There are, for instance, enzymes that modify histones by acetylation/deacetylation, phosphorylation, methylation, ubiquitination, or ADP-ribosylation. In addition, ATP-dependent proteins, termed chromatin remodeling factors, alter histone-DNA contacts and catalyze nucleosome mobility. It will thus be important to understand the relation between chromatin structure and the activity of each of the tens of thousands of genes in an organism.

To investigate the role of chromatin in transcriptional regulation, it is possible to use biochemical systems for the analysis of *in vitro* reconstituted chromatin. To this end, we previously developed a chromatin transcription system that is based on a crude chromatin assembly extract termed the S190 extract, which is derived from *Drosophila* embryos (14, 15). This S190-based chromatin transcription system has been used for the analysis of many transcription factors.

Although the S190 extract has been a reliable source of chromatin assembly activity, we have recently achieved the ATP-dependent assembly of chromatin with purified, recombinant chromatin assembly factors (16). In these reactions, periodic nucleosome arrays are assembled with purified recombinant ACF,¹ purified recombinant dNAP1, purified native core histones, plasmid DNA, and ATP. dNAP1 functions stoichiometrically as a core histone chaperone, whereas ACF acts catalytically in an ATP-dependent manner to mediate the deposition of histones onto DNA as well as to catalyze the formation of periodic nucleosome arrays.

In this work, we have used the purified chromatin assembly factors, instead of the S190 extract, to assemble chromatin for transcriptional analyses. With this new chromatin transcription system, we have found that the presence of acetyl-CoA at the time of transcription preinitiation complex assembly is essential for activation with chromatin templates.

EXPERIMENTAL PROCEDURES

Transcription Factors and DNA Templates—FLAG-tagged human estrogen receptor α (ER), His₆-tagged NF- κ B p65, and His₆-tagged p300 were synthesized in Sf9 cells and purified as described previously (17, 18). Purified Sp1 was obtained from Promega (Madison, WI). Gal4-VP16 was purified as described previously (19). Transcription reactions with NF- κ B p65 and Sp1 were carried out with a DNA template containing the IRF-1 promoter region (from –1312 to +39 relative to the RNA start site) (20). Transcription with the ER was performed with pERE, which contains four estrogen response elements upstream of the adenovirus E4 core promoter (17). Transcription with Gal4-VP16 was carried out with pGIE-0, which contains five Gal4 binding sites upstream of the adenovirus E4 core promoter (14).

Chromatin Assembly—The assembly of chromatin with purified recombinant ACF, purified recombinant dNAP1, purified *Drosophila* core histones, plasmid DNA, and ATP was performed as described by Ito *et al.* (16), except that the scale of the reactions was typically about five times larger than that used previously. The assembly of chromatin with the *Drosophila* S190 extract was carried out as described by Bulger and Kadonaga (21). The resulting chromatin samples were subjected to *in vitro* transcription analysis as well as to micrococcal nuclease digestion analysis to confirm the efficient assembly of periodic nucleosome arrays.

In Vitro Transcription—*In vitro* transcription reactions were per-

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¹ The abbreviations used are: ACF, ATP-utilizing chromatin assembly and remodeling factor; dNAP1, *Drosophila* nucleosome assembly protein 1; ER, estrogen receptor; NF- κ B, nuclear factor κ B; IRF-1, interferon regulatory factor-1; PIC, preinitiation complex; rDNA, ribosomal DNA; rNTP, ribonucleoside 5'-triphosphate.

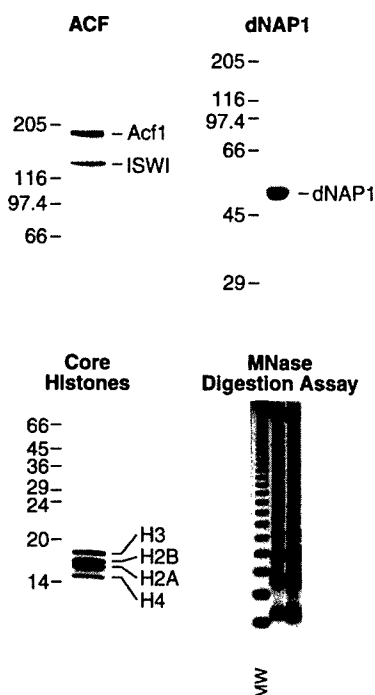


FIG. 1. Assembly of periodic nucleosome arrays with purified recombinant ACF and dNAP1. Purified recombinant ACF, purified recombinant dNAP1, and purified native core histones were analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The sizes of molecular mass markers (in kilodaltons) are indicated. Chromatin was assembled with the purified proteins, plasmid DNA, and ATP, and then subjected to micrococcal nuclease (MNase) digestion analysis. The mass markers (MW) in the left lane are the 123-base pair ladder (Life Technologies, Inc.). The samples in the middle and right lanes were obtained by digestion of the chromatin with different concentrations of micrococcal nuclease.

formed with HeLa nuclear extracts, as described previously (17, 18). With chromatin templates, the transcriptional activators, p300 (where indicated), and HeLa extract were added subsequent to the completion of chromatin assembly, except where noted otherwise. Acetyl-CoA (Sigma; catalog number A-2056) was typically used at a concentration of 9 μ M, but variation of the acetyl-CoA concentration from 3 to 300 μ M yielded essentially identical results (data not shown). Quantitation of the data was carried out with a PhosphorImager (Molecular Dynamics). All reaction conditions were performed in duplicate, and each experiment was performed a minimum of two (but typically, several) independent times to establish the reproducibility of the results.

RESULTS

The cloning of the Acf1 subunit of ACF led to the development of a purified chromatin assembly system that consists of defined components: purified recombinant ACF, purified recombinant dNAP1, purified native core histones, plasmid DNA, and ATP (16). The purified proteins are shown in Fig. 1. ACF consists of Acf1 and ISWI subunits and catalyzes the ATP-dependent deposition of histones onto DNA as well as the periodic spacing of nucleosomes (16). dNAP1 is a core histone chaperone that is a homomultimer of a polypeptide with a calculated molecular mass of ~43 kDa, although it migrates with a larger apparent mass on an SDS-polyacrylamide gel (22). The assembly of extended, periodic nucleosome arrays with the purified factors is shown in Fig. 1.

With this system, we compared the transcriptional properties of chromatin assembled with purified ACF + dNAP1 to those of chromatin assembled with the crude S190 extract. These experiments were performed as follows. First, plasmid DNA was assembled into chromatin with either purified ACF + dNAP1 or S190 extract, and then a nuclear extract was added as a source of the RNA polymerase II transcriptional machinery. When desired, sequence-specific activators and purified

human p300 were added after chromatin assembly and prior to the addition of the nuclear transcription extract.

In initial experiments with the Gal4-VP16 activator and the soluble nuclear fraction (a crude, undialyzed nuclear transcription extract derived from *Drosophila* embryos) (23, 24), we observed comparable levels of transcription with the recombinant ACF + dNAP1-assembled chromatin as with the S190-assembled chromatin (data not shown). These results with recombinant ACF and dNAP1 are similar to those that we had obtained previously with native ACF and dNAP1 in conjunction with Gal4-VP16 and the soluble nuclear fraction (25).

In contrast, when we performed transcription reactions with Gal4-VP16 and a standard HeLa nuclear extract (26) instead of the soluble nuclear fraction, there was robust transcription from the S190-assembled chromatin, but no detectable transcription from the ACF + dNAP1-assembled chromatin. To investigate the basis for the lack of Gal4-VP16-activated transcription with the ACF + dNAP1-assembled chromatin, we performed a series of experiments in which we tested the ability of proteins and protein fractions as well as nonprotein factors to stimulate transcription with the ACF + dNAP1-assembled chromatin and the HeLa extract. These studies led to the identification of acetyl CoA as a key, essential cofactor that is present in the S190 chromatin assembly extract (and presumably the soluble nuclear fraction) and is absent from our preparations of purified ACF and dNAP1.

This acetyl-CoA dependence of transcription was then tested with NF- κ B p65 + Sp1 (Fig. 2, top row) and human estrogen receptor α (Fig. 2, middle row) as well as Gal4-VP16 (Fig. 2, bottom row). With ACF + dNAP1-assembled chromatin, we observed a strong enhancement of transcription by acetyl-CoA that varied from 21- to 41-fold with a standard HeLa transcription extract and from 17- to 41-fold with HeLa extract supplemented with purified p300 coactivator (Fig. 2, left panels). In contrast, with S190-assembled chromatin, there was no significant change in transcriptional activity upon addition of acetyl-CoA (ranging from a 0.7- to 1.4-fold effect), presumably due to the presence of acetyl-CoA in the S190 extract (Fig. 2, middle panels). With naked DNA templates, we observed a slight increase in transcription upon addition of acetyl-CoA that varied from 1.2- to 2.5-fold (Fig. 2, right panels). In addition, trichostatin A, an inhibitor of histone deacetylases, did not enhance transcription in the absence of exogenously added acetyl-CoA with the ACF + dNAP1-assembled chromatin (data not shown). Thus, these findings collectively indicate that there is a general chromatin-specific requirement for acetyl-CoA for transcriptional activation.

It is also important to note that the exposure times of the three autoradiograms in each row of Fig. 2 are either identical (i.e. derived from the same autoradiogram) or nearly identical. Therefore, the levels of activated transcription from the ACF + dNAP1-assembled chromatin are comparable with those seen with S190-assembled chromatin or with naked DNA templates. Hence, there is robust transcription from the ACF + dNAP1-assembled chromatin in the presence of acetyl-CoA.

To investigate further the transcriptional properties of the ACF + dNAP1-assembled chromatin, we examined whether or not there is a greater amount of transcription when activators are added to naked DNA templates prior to chromatin assembly than when activators are added to chromatin that is previously assembled. It is possible, for instance, that the purified chromatin assembly system may lack nucleosome remodeling activities that are necessary for activators to bind to chromatin and/or to recruit coactivators and the basal transcriptional machinery. As shown in Fig. 3, we observed essentially identical levels of transcriptional activation by either NF- κ B p65 +

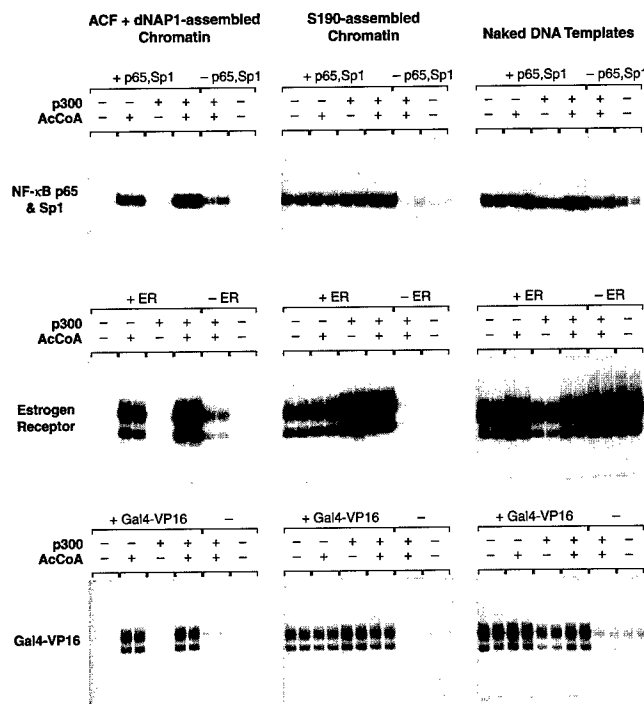


FIG. 2. Acetyl-CoA is required for transcription of chromatin assembled with purified ACF and dNAP1. The transcriptional properties of chromatin assembled with purified ACF and dNAP1 (left panels), chromatin assembled with crude S190 extract (center panels), or naked DNA (right panels) were tested with purified NF- κ B p65 + purified Sp1 (top row), purified estrogen receptor (ER) + 17 β -estradiol (middle row), or purified Gal4-VP16 (bottom row). Sequence-specific activators, purified human p300, and/or acetyl-CoA (AcCoA) were added, as indicated, subsequent to chromatin assembly and prior to *in vitro* transcription with a HeLa nuclear extract. The resulting transcripts were detected by primer extension analysis, and the reverse transcription products are shown. The final concentrations of acetyl-CoA, p300, NF- κ B p65, Sp1, estrogen receptor, 17 β -estradiol, and Gal4-VP16 in the transcription reaction media were 9 μ M, 15 nM, 60 nM, 9 nM, 4.5 nM, 30 nM, and 7.5 nM, respectively. For each row of reactions performed with a particular activator set (NF- κ B p65 + Sp1, estrogen receptor, or Gal4-VP16), the time of exposure of the autoradiograms is either identical or nearly identical. Therefore, for each activator, the autoradiograms reflect the relative amounts of transcription from the ACF + dNAP1-assembled chromatin relative to the S190-assembled chromatin and to naked DNA templates.

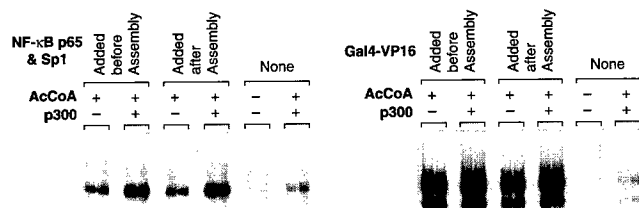


FIG. 3. Transcriptional activators function effectively with chromatin that is previously assembled with ACF and dNAP1. The transcriptional activators NF- κ B p65 + Sp1 (left panel) or Gal4-VP16 (right panel) were added either to naked DNA templates that were subsequently assembled into chromatin with purified ACF and dNAP1 ("Added before Assembly") or to chromatin templates that were previously assembled with purified ACF and dNAP1 ("Added after Assembly"). The resulting templates were then subjected to *in vitro* transcription analysis with a HeLa nuclear extract in the presence or absence of acetyl-CoA and/or purified p300, as noted. The transcripts were detected by primer extension analysis. The concentrations of reagents are as indicated in the legend to Fig. 2.

Sp1 or Gal4-VP16 when the activators were added either before or after chromatin assembly. Similar results were also obtained with the estrogen receptor (data not shown). Therefore, tran-

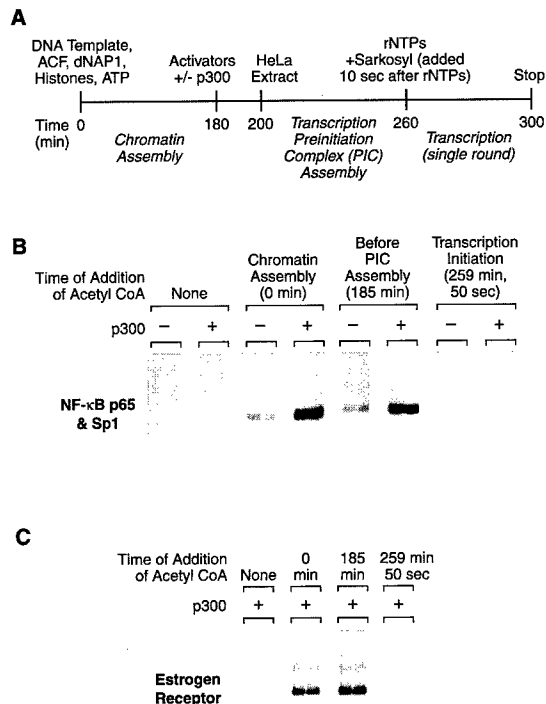


FIG. 4. Acetyl-CoA is required at the time of transcription preinitiation complex assembly. A, scheme for single round transcription analysis. The experimental procedure is described under "Results." B, single round transcription analysis with NF- κ B p65 and Sp1 and the human IRF-1 promoter. The time of addition of acetyl-CoA is according to the scheme shown in A. The presence or absence of purified exogenous p300 is indicated. C, single round transcription analysis with human estrogen receptor. The time of addition of acetyl-CoA is according to the scheme shown in A. The concentrations of reagents are as indicated in the legend to Fig. 2.

scriptional activators function effectively with chromatin that is previously assembled with ACF and dNAP1.

Last, to gain a better understanding of the acetyl-CoA effect, we sought to identify the step at which acetyl-CoA stimulates transcription. To this end, we performed single round transcription experiments, as outlined in Fig. 4A. In these experiments, chromatin assembly by ACF and dNAP1 was initiated at time = 0 min. At time = 180 min, activators (NF- κ B p65 + Sp1 or estrogen receptor) and purified p300 (where indicated) were added to the chromatin. Then, at time = 200 min, HeLa nuclear extract was added to allow the assembly of the transcription preinitiation complex (PIC). Next, at time = 260 min, the four ribonucleoside 5'-triphosphates (rNTPs) were added to initiate transcription. To limit transcription to a single round, the detergent Sarkosyl (0.2% w/v final concentration) was added 10 s after the rNTPs. Sarkosyl inhibits PIC assembly but not elongation of transcriptionally engaged RNA polymerase II (27–29).

In this manner, single round transcription experiments were carried out in which acetyl-CoA was added at different times in the chromatin assembly and transcription processes. The results obtained with NF- κ B p65 + Sp1 at the IRF-1 promoter are shown in Fig. 4B. First, when acetyl-CoA was added at the beginning of the chromatin assembly process (at time = 0 min), we observed a strong stimulation of transcription, as seen in Fig. 2. Similarly, there was also a strong stimulation of transcription when acetyl-CoA was added after chromatin assembly and prior to PIC assembly (at time = 185 min). These results indicate that acetyl-CoA is not required during chromatin assembly for transcriptional activation. In contrast, when acetyl-

CoA was added subsequent to PIC incubation and prior to transcription initiation (at time = 259 min 50 s), we did not observe transcriptional activation in either the absence or the presence of exogenously added p300. Hence, these findings indicate that acetyl-CoA is required at the time of PIC assembly. In addition, essentially identical results were obtained with the estrogen receptor (Fig. 4C).

It was also possible, however, that the lack of activation by acetyl-CoA upon addition after PIC incubation (at time = 259 min 50 s) was due to the inactivation of an acetyltransferase during the 60-min PIC incubation period (as shown in Fig. 4A). Thus, as a control, we carried out reactions in which the HeLa extract was preincubated separately from the chromatin (which contained p300 and activators) for 75 min at 30 °C and then subsequently combined with the chromatin to initiate PIC assembly. Under these conditions, we still observed a strong dependence upon acetyl-CoA for transcription of ACF + dNAP1-assembled chromatin (data not shown). We therefore conclude that acetyl-CoA is required at the time of PIC assembly for the efficient transcription of chromatin templates.

DISCUSSION

We have used a defined, purified chromatin assembly system to generate periodic nucleosome arrays for the analysis of chromatin structure and transcriptional regulation. In these experiments, we found that acetyl-CoA is essential for transcriptional activation with chromatin templates and that acetyl-CoA is required at the time of PIC assembly.

Given the extensive acetylation of core histones and the chromatin specificity of the acetyl-CoA dependence, it is reasonable to postulate that the potent stimulation by acetyl-CoA (e.g. 41-fold enhancement with the estrogen receptor) is due to histone acetylation. On the other hand, it is possible that the acetylation of a transcription factor will enhance its ability to function with chromatin. In this regard, the acetylation of sequence-specific DNA binding factors (see, for example, Refs. 30–32), the ACTR transcriptional coactivator (33), and the basal transcription factors TFIIE and TFIIF (34) have been observed. Moreover, it has been found that acetyl-CoA can enhance the transcription of naked DNA (greater than 5-fold) as well as the binding of TFIID to promoter DNA in the absence of histones (35). (Note that we observe a more modest 1.2–2.5-fold increase in transcription by acetyl-CoA with naked DNA templates (Fig. 2, right panels).) Thus, in our experiments, protein acetylation might affect not only the properties of components of chromatin, but also the activity of transcription factors and coactivators that function with chromatin.

For transcription with chromatin templates assembled with recombinant ACF and dNAP1, we observed a strong dependence upon acetyl-CoA with a standard HeLa nuclear extract (26) (Fig. 2, left panels). In contrast, only a 2–3-fold enhancement of transcription by acetyl-CoA was seen with a standard HeLa transcription extract in conjunction with a nucleosomal template consisting of a promoter-containing dinucleosome embedded in an array of nucleosomes in tandem 5 S rDNA repeats (36, 37). In the same series of experiments, however, the magnitude of acetyl-CoA stimulation was increased upon the addition of any one of four different acetyltransferase-containing complexes. Then, in other studies with a chromatin template consisting of a promoter embedded in a tandem 5 S rDNA array, Kundu *et al.* (38) have described p300-dependent, Gal4-VP16-activated transcription that is stimulated by acetyl-CoA. These results suggest that p300/CBP and/or HAT complexes such as SAGA, NuA3, NuA4, or Ada may contribute to the acetyl-CoA requirement that we have observed. Yet, unlike the chromatin in the tandem 5 S rDNA arrays, the ACF + dNAP1-assembled chromatin exhibits a strong dependence upon

acetyl-CoA with a standard HeLa extract that is not supplemented with exogenously added acetyltransferases.

In the future, we hope to use the ACF + dNAP1-based chromatin transcription system to gain a better understanding of the essential acetylation event(s) at the time of PIC assembly. Importantly, the efficiency of transcription from the ACF + dNAP1-assembled chromatin is comparable with that obtained with either S190-assembled chromatin or with naked DNA templates. Thus, the ACF + dNAP1-based chromatin transcription system is likely to be of general utility for the analysis of the function of a broad range of transcriptional regulators at their downstream target genes. These studies should yield new insights into fundamental aspects of the complex and fascinating mechanisms of gene regulation.

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